Data Evaluation Report on the Toxicity of Bitrex® (Denatonium benzoate) to Algae (*Nitzschia palea*)

Data Requirement:

DP Barcode: 340828
MRID: 47135819
Guideline: OCSPP 850.4500

Test material: Bitrex®
Common name: Denatonium benzoate
Chemical name: IUPAC: Lignocaine benzyl benzoate N-(2-((2,6-dimethyl phenyl) amino)-2-oxoethyl)-N, N-diethyl-benzenemethanaminium benzoate
CAS name: Not reported
CAS No.: 3734-33-6
Synonyms: None reported

Purity: Not reported

Primary Reviewer: John Marton
Staff Scientist, Cambridge Environmental, Inc.

Signature:
Date: 09/10/07

Secondary Reviewer: Teri S. Myers
Senior Scientist, Cambridge Environmental, Inc.

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Date: 09/26/07

Primary Reviewer: Michael Lowit
Ecologist, EPA/OCSPP/OPP/EFED/ERB1

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Date: 2/28/14

Secondary Reviewer: Robin Sternberg
Wildlife Biologist, EPA/OCSPP/OPP/EFED/ERB1

Signature:
Date: 2/28/14

PC Code: 009106

Date Evaluation Completed: 2/28/14

CITATION: Peterson, Gitte I. 1991. Growth Inhibition Test of Bitrex® With *Nitschia palea* (Algae). Unpublished study performed by Water Quality Institute, Science Park Horsholm, Denmark. Laboratory report number 308131/416. Study sponsored by Gori All-Wood International, Denmark. Study submitted by Johnson Matthey Macfarlan Smith, Edinburgh, United Kingdom. Study completed September 11, 1991.

DISCLAIMER: This document provides guidance for EPA and PMRA reviewers on how to complete a data evaluation record after reviewing a scientific study concerning the acute toxicity of a pesticide to aquatic nonvascular plants. It is not intended to prescribe conditions to any external party for conducting this study nor to establish absolute criteria regarding the assessment of whether the study is scientifically sound and whether the study satisfies any applicable data requirements. Reviewers are expected to review and to determine for each study, on a case-by-case basis, whether it is scientifically sound and provides sufficient information to satisfy applicable data requirements. Studies that fail to meet any of the conditions may be accepted, if appropriate; similarly, studies that meet all of the conditions may be rejected, if appropriate. In sum, the reviewer is to take into account the totality of factors related to the test methodology and results in determining the acceptability of the study.

Data Evaluation Report on the Acute Toxicity of Bitrex® (Denatonium benzoate) to Algae (*Nitzschia palea*)

EXECUTIVE SUMMARY

In a 70.5-hour toxicity study, cultures of *Nitzschia palea* were exposed to Bitrex® (denatonium benzoate) at nominal concentrations of 0 (control), 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 mg Bitrex/L under static conditions. The NOAEC = 5 mg/L for yield, growth rate, and area under the growth curve. The IC_{50} was between 5 and 10 mg/L for all endpoints; it could not be estimated for yield due to a poor fit but was 5.7 mg/L for area under the growth curve and 6.1 mg/L for growth rate. No compound-related phytotoxic effects were reported.

This study is classified as **Supplemental (qualitative use)** and does not satisfy the guideline requirement for a freshwater diatom toxicity test because it was conducted for 70.5 hr (96 hr is the minimum requirement) using a non-recommended species. Furthermore, the endpoints should be used only qualitatively due to other deviations including but not limited to the lack of logarithmic growth after 45.5 hr, low initial cell density, and use of three replicates instead of four.

Results Synopsis

Yield (0-70.5 hr)
IC_{50} = 5.0 to 10.0 mg/L   95% C.I. = N/A
NOAEC = 5.0 mg/L

Growth Rate (0-45.5 hr)
IC_{05} = 4.8 mg/L   95% C.I. = 4.5-5.1 mg/L
IC_{50} = 6.1 mg/L   95% C.I. = 5.8-6.5 mg/L
NOAEC = 5.0 mg/L
Probit Slope = 15.4±1.50

Area Under the Growth Curve (0-70.5 hr)
IC_{05} = 3.9 mg/L   95% C.I. = 3.3-4.6 mg/L
IC_{50} = 5.7 mg/L   95% C.I. = 5.2-6.3 mg/L
NOAEC = 5.0 mg/L
Probit Slope = 9.88±1.39

Endpoints affected: yield, growth rate, and area under the growth curve

*Toxicity is reported in terms of nominal concentrations of Bitrex. Nominal concentrations are taken at face value without adjustment for purity (see Study Deficiencies).

Data Evaluation Report on the Acute Toxicity of Bitrex® (Denatonium benzoate) to Algae (*Nitzschia palea*)

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: This study was conducted following guidelines outlined in OECD Guideline 201 "Alga, Growth Inhibition Test", which corresponds closely to the ISO Draft International Standard ISO/DIS 8692 "Water Quality-Algal Growth Inhibition Test." The following deviations from OCSPP 850.4500 were noted:

1. The purity of the test material was not reported.
2. The duration of the test (70.5 hr) was shorter than recommended (96 hr).
3. The test organism, *Nitzschia palea*, is not one of the recommended species.
4. Information was not provided about the acclimation period.
5. Full details of the growth media were not provided.
6. The results of a periodic screening analysis of the dilution water were not included.
7. The method of introduction of the test material into the test solutions was not described.
8. Information was not provided about the algae source and age.
9. Information was not provided about the assignment of treatments or positioning of test vessels in the growth chamber.
10. Three replicates per treatment level were tested; four replicates are recommended.
11. Initial cell density was 4300 cells/mL; a minimum of 10,000 cells/mL is recommended.
12. Photoperiod was not reported.
13. Information was not provided about how and when measurements were made for temperature and light intensity.
14. Test concentrations were not analytically verified.

Collectively the deviations impact the acceptability of the study (#2, #3, #9, #10, #11, and #14 were the most important). In addition, although logarithmic growth was achieved, growth slowed considerably after 45.5 hr.

COMPLIANCE: Signed and dated No Data Confidentiality, GLP and Quality Assurance statements were provided. This study was conducted in compliance with principles of Good Laboratory Practice according to OECD codes of GLP, May 1981, Doc C (81)30 (Final).

A. MATERIALS

1. Test material Bitrex® (Denatonium benzoate)

Description: White granule

Lot No./Batch No. : Not reported

Purity: Not reported

Stability of compound under test conditions: Analytical verification was not conducted during this study. However, chemical analysis was performed on samples from a preliminary test with *Daphnia magna* of concentrations (50 and 100 mg/L) collected at 0, 24, 48, and 70 hr after the start of the test. The results indicated that Bitrex® was stable over a 70-hr test period (recoveries after 70 hr were 101% and 99.8%

of initial (t_0) recoveries at the 50 and 100 mg/L levels, respectively).

Storage conditions of test chemicals: Not reported

Physicochemical properties of Bitrex®

Parameter	Values	Comments
Water solubility	45 g/L	Temperature not specified
Vapor pressure	Not reported	
UV absorption	Not reported	
pKa	Not reported	
Kow	Not reported	

2. Test organism

     Name: *Nitzschia palea*

     Navicula pelliculosa is the recommended freshwater diatom species.

Strain:	Not reported
Source:	Not reported
Age of inoculum:	Not reported; however, it was stated that cultures were growing exponentially.
Method of cultivation:	Synthetic medium prepared from Millipore water according to the ISO/DIS Standard 8692

B. STUDY DESIGN

     1. Experimental Conditions

       a. Range-finding study

       None reported

b. Definitive study

Table 1. Experimental Parameters

Parameter	Details	Remarks
		Criteria
Acclimation period	Not reported	
Culturing media and conditions (same as test or not)	Not reported	
Health (any mortality observed)	The only information regarding the culture prior to test initiation was that it was growing exponentially.	*Six weeks of culturing is recommended for cultures not previously maintained at the test facility. Algal inoculum should be from logarithmically-growing stock cultures.*
Test system Static/static renewal	Static	
Renewal rate for static renewal	N/A	
Incubation facility	Not reported	
Duration of the test	70.5 hours	It was noted that exponential growth stopped after 45.5 hours in all test vessels, including the control vessels.
		96 hr
Test vessel Material *(glass/stainless steel)* Size Fill volume	Glass 250 mL 100 mL	Glass beakers were used as caps.
		125-500mL
Details of growth medium pH at test initiation pH at test termination Chelator used Carbon source	Freshly-prepared synthetic medium prepared from Millipore water according to the ISO/DIS Standard 8692 7.8±0.2 7.6-7.8 Not reported Not reported	*Formulation and sterilization of nutrient medium should conform to current ASTM recommendations for algal toxicity tests. Chelating agents should be included.*

Parameter	Details	Remarks
		Criteria
If non-standard nutrient medium was used, detailed composition provided (Yes/No)	No	
Dilution water source/type pH water pretreatment (if any) total organic carbon particulate matter metals pesticides chlorine	Millipore water 7.8±0.2 Not reported Not reported Not reported Not reported Not reported Not reported	*Reagent quality water. The dilution water source used to prepare media should be periodically analyzed to document and characterize the hardness, alkalinity, pH, conductivity, total organic carbon (TOC) or chemical oxygen demand magnitude and variability, and to ensure that pesticides, PCBs and toxic metals are not present at concentrations that are considered toxic.*
Indicate how the test material is added to the medium (added directly or used stock solution)	Not reported	
Aeration or agitation	Not reported	
Initial cells density	4.3×10^3 cells/mL	
		10,000 cells/mL for P. subcapitata and S. costatum. At least 10,000 cells/mL for other species.
Number of replicates Control Solvent control Treatments	 5 N/A 3	Two blank vessels containing no algae were also prepared as a baseline for biomass measurements via fluorescence.
		Minimum of four replicates
Test concentrations Nominal Measured	 0 (control), 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 mg Bitrex/L Not determined	 *Minimum of five test concentrations in a geometric progression of twofold at a minimum.*

Parameter	Details	Remarks
		Criteria
Solvent (type, percentage, if used)	A solvent was not used.	
Method and interval of analytical verification	Analytical verification was not conducted.	
Test conditions Temperature Photoperiod Light intensity and quality	19±2°C Not reported $7.0 \pm 0.2 \times 10^{15}$ quanta x cm^{-2} x sec^{-1}	Recommendations not established for *Nitzschia palea*
Reference chemical (if used)	A reference chemical was not used.	
Other parameters, if any	None	

2. Observations

Table 2. Observation parameters

Parameters	Details	Remarks
		Criteria
Parameters measured including the growth inhibition/other toxicity symptoms	-Biomass (measured as fluorescence) -Growth rate -Area under the growth curve	*EPA recommends the growth of the algae expressed as the cell count per mL, biomass per volume, or degree of growth as determined by spectrophotometric means.*

Parameters	Details	Remarks
		Criteria
Measurement technique for cell density and other end points	Biomass was determined by subtracting the fluorescence measurements for the blanks (vessels containing no algae) from the fluorescence measurements for the treatment vessels. Growth rate and area under the growth curve were calculated from biomass. The growth rate was calculated by log linear regression on biomass versus time and the biomass growth as the area under the growth curve as described in the ISO/DIS Standard 8692.	*EPA recommends the measurement of cell counts or chlorophyll a.*
Observation intervals	23, 45.5 and 70.5 hr	*Every 24 hours*
Other observations, if any	Exponential growth during the 45.5-70.5 hr period stopped in all test vessels, including the controls. Therefore, growth rate was based on the 0 to 45.5 hr time period.	
Indicate whether there was an exponential growth in the control	Yes, at 45.5 hr of exposure, biomass increased approximately 40X from the start of the test.	*Cell counts in the controls should increase by a factor of at least 100 times for P. subcapitata and a factor of at least 30 times for S. costatum by test termination (i.e., logarithmic growth in the controls).*
Were raw data included?	Yes	

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II. RESULTS and DISCUSSION

A. INHIBITORY EFFECTS

Growth rate (0-45.5 hours) averaged 1.969 day^{-1} in the negative control and 1.912, 2.153, 2.102, 2.058, 2.026, 1.847, and 0.001 day^{-1} in the nominal 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 mg/L treatment groups, respectively. Corresponding inhibition relative to the negative control was 2.9, -9.4, -6.8, -4.5, -2.9, 6.2, and 99.9%. The study author reported an EC_{50} of 5-10 mg/L.

Area under the growth curve (0-70.5 hours) averaged 904.7 in the negative control and 981.0, 1213.4, 1139.1, 1030.9, 1058.7, 749.7, and 8.8 in the nominal 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 mg/L treatment groups. Corresponding inhibition relative to the negative control was -8.4, -34.1, -25.9, -14.0, -17.0, 17.1 and 99.0% relative to the negative control, respectively. The study author reported an EC_{50} of 5-10 mg/L.

No abnormal observations were reported.

Table 3. Effect of Bitrex on growth of *Nitzchia palea*

Nominal Concentrations mg Bitrex/L	Initial cell Density (x10^3 cells/mL)	Mean Growth Rate per day (0-45.5 hr)	Growth Rate % Inhibition[a]	Area Under the Growth Curve (0-70.5 hr) (x10^4)	Area Under the Growth Curve % Inhibition[a]
Control	4.3	1.969	N/A	904.7	N/A
0.1	4.3	1.912	2.9	981.0	-8.4
0.2	4.3	2.153	-9.4	1213.4	-34.1
0.5	4.3	2.102	-6.8	1139.1	-25.9
1.0	4.3	2.058	-4.5	1030.9	-14.0
2.0	4.3	2.026	-2.9	1058.7	-17.0
5.0	4.3	1.847	6.2	749.7	17.1
10.0	4.3	0.001	99.9	8.8	99.0
Reference chemical (if used)	N/A	N/A	N/A	N/A	N/A

[a] Reviewer-estimated percent inhibition compared to the negative control. Negative percent inhibition indicates promoted growth.
N/A- not applicable

Table 4. Statistical endpoints

Statistical Endpoint	Growth Rate	Area Under the Growth Curve
NOAEC or EC_{05} (mg/L)	Not Reported	Not Reported
EC_{50} (mg/L)	5-10	5-10
Reference chemical, if used	N/A	N/A

N/A- Not Applicable

B. REPORTED STATISTICS

A computer program (TOXEDO, developed by VKI) was used to calculate the concentration inhibiting growth (EC_{10} and EC_{50}). Calculations were carried out assuming a logarithmic normal distribution of data. The curve fitting was carried out using a weighted linear regression analysis on (linear) probit transformed data. Estimation of curve parameters by regression analysis was supplied with a reliability measure of the applied linear model. Inverse estimation was used to determine values of EC-statistics and to calculate confidence limits.

C. VERIFICATION OF STATISTICAL RESULTS

The reviewer analyzed growth rate and area under the growth curve for statistical significance. Prior to determining the NOAEC, both data sets were tested for normality using the Chi-square and Shapiro-Wilks tests and homogeneity of variance using the Hartley and Bartlett's tests. The growth rate data did not meet the assumption of homogeneity of variance; therefore, the NOAEC was determined using the non-parametric Kruskal-Wallis test. The area under the growth curve data met the assumptions of parametric statistics; therefore, the NOAEC was determined using the parametric Bonferroni and Williams tests. All NOAEC determinations were made via Toxstat statistical software. The ICx values were determined using probit analysis via Nuthatch statistical software. All analyses were conducted using nominal concentrations.

The original (contractor) review did not analyze yield (reported as fluorescence). Yield was analyzed using CETIS v. 1.8.7.12 (the currently used statistical software). Data satisfied the assumptions of normality and homogeneity of variance. The NOAEC and LOAEC were determined using Williams test because the data met assumptions (normality and equal variance) and there was an overall monotonic trend (statistical results are the same for the "no trend" analysis). The IC_{50} was not calculable due to a poor fit. Visual inspection of the data indicates that the IC_{50} was between 5 and 10 mg/L.

Yield (0-70.5 hr)
IC_{50} = 5.0 to 10.0 mg/L   95% C.I. = N/A
NOAEC = 5.0 mg/L

Growth Rate (0-45.5 hr)
IC_{05} = 4.8 mg/L   95% C.I. = 4.5-5.1 mg/L
IC_{50} = 6.1 mg/L   95% C.I. = 5.8-6.5 mg/L
NOAEC = 5.0 mg/L
Probit Slope = 15.4±1.50

Area Under the Growth Curve (0-70.5 hr)
IC_{05} = 3.9 mg/L   95% C.I. = 3.3-4.6 mg/L
IC_{50} = 5.7 mg/L   95% C.I. = 5.2-6.3 mg/L

NOAEC = 5.0 mg/L
Probit Slope = 9.88±1.39

*Toxicity is reported in terms of nominal concentrations of Bitrex. Nominal concentrations are taken at face value without adjustment for purity (see Study Deficiencies).

D. STUDY DEFICIENCIES

Any potential effects after 45.5 hr cannot be discerned because there was minimal growth between 45.5 and 70.5 hr. Therefore, toxicity values may have been lower if logarithmic growth had continued through 96 hr (minimum recommended test duration).

Purity of the test material should have been reported, and concentration of the test substance in the chambers should have been measured during the definitive test. However, preliminary testing with Bitrex® showed that measured concentrations were about 102% of nominal at $t = 0$ (preliminary testing was in conjunction with a *Daphnia magna* test). This suggests that nominal test concentrations in the *Nitzschia palea* study can be taken at face value without adjustment for purity. In preliminary testing, concentrations of Bitrex® were stable under static conditions for up to 70 hr (101% and 99.8% of initial (t_0) recoveries at the 50 and 100 mg/L levels, respectively). This suggests that concentrations were likely stable during the test with *Nitzschia palea*. However, there is some uncertainty because the *Daphnia magna* testing conditions were not reported and may not have been the same as those in the *Nitzschia palea* study.

The study would have benefitted from additional replication given that a statistically significant effect could not be detected at the 5 mg/L treatment level for a 17% inhibition of area under the growth curve and a 10% inhibition of yield.

E. REVIEWER COMMENTS

The physiochemical properties of the test material were not reported.

At test termination, the coefficient of variation for mean control yield and specific growth rate was less than 15%.

The in-life portion of the definitive study was conducted from August 13 to August 16, 1991.

F. CONCLUSIONS

This study is classified as **Supplemental (qualitative use)** and does not satisfy the guideline requirement for a freshwater diatom toxicity test because it was conducted for 70.5 hr (96 hr is the minimum requirement) using a non-recommended species. Furthermore, the endpoints should be used only qualitatively due to other deviations including but not limited to the lack of logarithmic growth after 45.5 hr, low initial cell density, and use of three replicates instead of four.

Yield (0-70.5 hr)
IC_{50} = 5.0 to 10.0 mg/L   95% C.I. = N/A
NOAEC = 5.0 mg/L

Growth Rate (0-45.5 hr)
IC_{05} = 4.8 mg/L   95% C.I. = 4.5-5.1 mg/L
IC_{50} = 6.1 mg/L   95% C.I. = 5.8-6.5 mg/L
NOAEC = 5.0 mg/L

Probit Slope = 15.4±1.50

Area Under the Growth Curve (0-70.5 hr)
IC_{05} = 3.9 mg/L   95% C.I. = 3.3-4.6 mg/L
IC_{50} = 5.7 mg/L   95% C.I. = 5.2-6.3 mg/L
NOAEC = 5.0 mg/L
Probit Slope = 9.88±1.39

Endpoints affected: yield, growth rate, and area under the growth curve

*Toxicity is reported in terms of nominal concentrations of Bitrex. Nominal concentrations are taken at face value without adjustment for purity (see Study Deficiencies).

III. REFERENCES

OECD Guideline for Testing of Chemicals No. 201. 1984. "Alga, Growth Inhibition Test."

ISO/Draft International Standard 8692. 1987. "Water quality- Algal growth inhibition test."

Kusk, K.O., Sorensen, P.S. and Larsen, P.D.B. 1988. TOXEDO. Program for statistical estimation of EC-values based on experimental data from ecotoxicological assays. Water Quality Institute, ATV, 11 Agern Alle, DK-2970 Horsholm, Denmak.

APPENDIX I. OUTPUT OF REVIEWER'S STATISTICAL VERIFICATION

```
Growth rate, mg/L; 0-45.5 hours
File: 5819gr      Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies
--------------------------------------------------------------------------

INTERVAL     <-1.5     -1.5 to <-0.5    -0.5 to 0.5    >0.5 to 1.5    >1.5
             _____     _____    _____    _____    _____

EXPECTED     1.742        6.292            9.932          6.292        1.742
OBSERVED     0            8                10             8            0

--------------------------------------------------------------------------
Calculated Chi-Square goodness of fit test statistic =   4.4118
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.




Growth rate, mg/L; 0-45.5 hours
File: 5819gr      Transform: NO TRANSFORMATION

Shapiro Wilks test for normality
--------------------------------------------------------------------------

D =    0.164

W =    0.972

Critical W (P = 0.05) (n = 26) = 0.920
Critical W (P = 0.01) (n = 26) = 0.891
--------------------------------------------------------------------------

Data PASS normality test at P=0.01 level. Continue analysis.




Growth rate, mg/L; 0-45.5 hours
File: 5819gr      Transform: NO TRANSFORMATION

Hartley test for homogeneity of variance
Bartletts test for homogeneity of variance
--------------------------------------------------------------------------

These two tests can not be performed because at least one group has
zero variance.

Data FAIL to meet homogeneity of variance assumption.
Additional transformations are useless.

--------------------------------------------------------------------------

Growth rate, mg/L; 0-45.5 hours
File: 5819gr       Transform: NO TRANSFORMATION

          KRUSKAL-WALLIS ANOVA BY RANKS  -  TABLE 1 OF 2
--------------------------------------------------------------------------
                        TRANSFORMED   MEAN CALCULATED IN      RANK
```

```
GROUP     IDENTIFICATION        MEAN         ORIGINAL UNITS         SUM
-----   ----------------      ----------    ------------------   ----------
  1        neg control         1.969                1.969         65.000
  2               0.1           1.912                1.912         26.000
  3               0.2           2.153                2.153         68.000
  4               0.5           2.102                2.102         63.000
  5               1.0           2.059                2.059         50.000
  6               2.0           2.026                2.026         50.000
  7               5.0           1.847                1.847         23.000
  8              10.0           0.001                0.001          6.000
-----------------------------------------------------------------------------


   Calculated H Value =  17.993     Critical H Value Table =  14.070
   Since  Calc H > Crit H  REJECT Ho:All groups are equal.



Growth rate, mg/L; 0-45.5 hours
File: 5819gr       Transform: NO TRANSFORMATION

 DUNNS MULTIPLE COMPARISON -  KRUSKAL-WALLIS  -  TABLE 2 OF 2
-----------------------------------------------------------------------------
                                              GROUP
                   TRANSFORMED  ORIGINAL   0 0 0 0 0 0 0 0
GROUP  IDENTIFICATION   MEAN       MEAN     8 7 2 1 6 5 4 3
-----  --------------- ---------- ---------  - - - - - - - -
  8              10.0     0.001      0.001   \
  7               5.0     1.847      1.847   . \
  2               0.1     1.912      1.912   . . \
  1        neg control    1.969      1.969   . . . \
  6               2.0     2.026      2.026   . . . . \
  5               1.0     2.059      2.059   . . . . . \
  4               0.5     2.102      2.102   . . . . . . \
  3               0.2     2.153      2.153   * . . . . . . \
-----------------------------------------------------------------------------
```

* = significant difference (p=0.05)   . = no significant difference
Table q value (0.05,8) =   3.124   Unequal reps - multiple SE values

Estimates of EC%
```
-------------------------------------------------------------------------
Parameter   Estimate     95% Bounds        Std.Err.  Lower Bound
                       Lower     Upper                /Estimate
EC5           4.8       4.5       5.1       0.014      0.94
EC10          5.0       4.7       5.4       0.013      0.94
EC25          5.5       5.2       5.8       0.012      0.95
EC50          6.1       5.8       6.5       0.012      0.94

        Slope =    15.4  Std.Err. =    1.50
```

Goodness of fit: p =   0.056 based on DF=   5.0   18.
```
-------------------------------------------------------------------------
5819GR : Growth rate, mg/L; 0-45.5 hours
-------------------------------------------------------------------------
Observed vs. Predicted Treatment Group Means
-------------------------------------------------------------------------
    Dose     #Reps.     Obs.     Pred.      Obs.      Pred.     %Change
                        Mean     Mean      -Pred.    %Control

    0.00      5.00      1.97     2.03     -0.0614     100.       0.00
```

```
   0.100      3.00      1.91      2.03     -0.118      100.   2.19e-14
   0.200      3.00      2.15      2.03      0.123      100.   2.19e-14
   0.500      3.00      2.10      2.03      0.0721     100.   2.19e-14
    1.00      3.00      2.06      2.03      0.0291     100.   2.19e-14
    2.00      3.00      2.03      2.03     -0.00420    100.   4.09e-12
    5.00      3.00      1.85      1.85      7.39e-13   91.0   9.01
   10.0       3.00      0.00100   0.00100 -3.38e-08   0.0493  100.
```

Area under the growth curve, mg/L; 0-70.5 hours
File: 5819gc   Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies
----

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.742	6.292	9.932	6.292	1.742
OBSERVED	0	9	8	9	0

Calculated Chi-Square goodness of fit test statistic =   6.1908
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.

Area under the growth curve, mg/L; 0-70.5 hours
File: 5819gc   Transform: NO TRANSFORMATION

Shapiro Wilks test for normality

D = 309733.615

W =   0.944

Critical W (P = 0.05) (n = 26) = 0.920
Critical W (P = 0.01) (n = 26) = 0.891

Data PASS normality test at P=0.01 level. Continue analysis.

Area under the growth curve, mg/L; 0-70.5 hours
File: 5819gc   Transform: NO TRANSFORMATION

Hartley test for homogeneity of variance

Calculated H statistic (max Var/min Var) = 415.50
Closest, conservative, Table H statistic = 2063.0 (alpha = 0.01)

Used for Table H ==>   R (# groups) = 8,   df (# reps-1) =   2
Actual values   ==>   R (# groups) = 8,   df (# avg reps-1) = 2.25
                                                (average df used)

Data PASS homogeneity test. Continue analysis.

NOTE: This test requires equal replicate sizes. If they are unequal
      but do not differ greatly, the Hartley test may still be used
      as an approximate test (average df are used).

Area under the growth curve, mg/L; 0-70.5 hours
File: 5819gc   Transform: NO TRANSFORMATION

Bartletts test for homogeneity of variance

Calculated B statistic =   10.35
Table Chi-square value =   18.48 (alpha = 0.01)
Table Chi-square value =   14.07 (alpha = 0.05)

Average df used in calculation ==> df (avg n - 1) =   2.25
Used for Chi-square table value ==> df (#groups-1) =   7

Data PASS homogeneity test at 0.01 level. Continue analysis.

NOTE: If groups have unequal replicate sizes the average replicate size is
      used to calculate the B statistic (see above).

Area under the growth curve, mg/L; 0-70.5 hours
File: 5819gc   Transform: NO TRANSFORMATION

                                ANOVA TABLE

SOURCE   DF   SS   MS   F

Between   7   3058910.987   436987.284   25.395

Within (Error)   18   309733.615   17207.423

Total   25   3368644.601

  Critical F value =   2.58 (0.05,7,18)
  Since F > Critical F REJECT Ho:All groups equal

Area under the growth curve, mg/L; 0-70.5 hours
File: 5819gc   Transform: NO TRANSFORMATION

    BONFERRONI T-TEST   -   TABLE 1 OF 2   Ho:Control<Treatment
----
                           TRANSFORMED   MEAN CALCULATED IN
GROUP   IDENTIFICATION   MEAN   ORIGINAL UNITS   T STAT SIG
----- ------------------   ----------   ------------------   ------ ---
  1   neg control   904.680   904.680
  2   0.1   981.033   981.033   -0.797
  3   0.2   1213.367   1213.367   -3.222
  4   0.5   1139.100   1139.100   -2.447
  5   1.0   1030.900   1030.900   -1.318
  6   2.0   1058.667   1058.667   -1.607

```
    7                    5.0    749.700            749.700          1.618
    8                   10.0      8.833              8.833          9.351  *
-----------------------------------------------------------------------------
Bonferroni T table value =  2.71     (1 Tailed Value, P=0.05,  df=18,7)
```

```
Area under the growth curve, mg/L; 0-70.5 hours
File: 5819gc       Transform: NO TRANSFORMATION

        BONFERRONI T-TEST   -   TABLE 2 OF 2          Ho:Control<Treatment
-----------------------------------------------------------------------------
                           NUM OF   Minimum Sig Diff  % of    DIFFERENCE
GROUP     IDENTIFICATION    REPS    (IN ORIG. UNITS)  CONTROL  FROM CONTROL
-----  --------------------  -------  ----------------  -------  -----------
  1          neg control    5
  2                  0.1    3           259.805        28.7      -76.353
  3                  0.2    3           259.805        28.7     -308.687
  4                  0.5    3           259.805        28.7     -234.420
  5                  1.0    3           259.805        28.7     -126.220
  6                  2.0    3           259.805        28.7     -153.987
  7                  5.0    3           259.805        28.7      154.980
  8                 10.0    3           259.805        28.7      895.847
-----------------------------------------------------------------------------
```

```
Area under the growth curve, mg/L; 0-70.5 hours
File: 5819gc       Transform: NO TRANSFORMATION

        WILLIAMS TEST  (Isotonic regression model)   TABLE 1 OF 2
-----------------------------------------------------------------------------
GROUP                              ORIGINAL     TRANSFORMED     ISOTONIZED
          IDENTIFICATION     N       MEAN          MEAN           MEAN
------  --------------------  ---  -----------  -----------   -----------
  1          neg control    5     904.680        904.680       1039.630
  2                  0.1    3     981.033        981.033       1039.630
  3                  0.2    3    1213.367       1213.367       1039.630
  4                  0.5    3    1139.100       1139.100       1039.630
  5                  1.0    3    1030.900       1030.900       1039.630
  6                  2.0    3    1058.667       1058.667       1039.630
  7                  5.0    3     749.700        749.700        749.700
  8                 10.0    3       8.833          8.833          8.833
-----------------------------------------------------------------------------
```

```
Area under the growth curve, mg/L; 0-70.5 hours
File: 5819gc       Transform: NO TRANSFORMATION

        WILLIAMS TEST  (Isotonic regression model)   TABLE 2 OF 2
-----------------------------------------------------------------------------
                    ISOTONIZED   CALC.     SIG     TABLE      DEGREES OF
    IDENTIFICATION     MEAN     WILLIAMS  P=.05   WILLIAMS     FREEDOM
--------------------  -----------  ----------  -----  -----------  -------------
       neg control   1039.630
               0.1   1039.630    1.409              1.73       k= 1, v=18
               0.2   1039.630    1.409              1.82       k= 2, v=18
               0.5   1039.630    1.409              1.85       k= 3, v=18
               1.0   1039.630    1.409              1.86       k= 4, v=18
               2.0   1039.630    1.409              1.87       k= 5, v=18
```

```
                   5.0    749.700     1.618                  1.87      k= 6, v=18
                  10.0      8.833     9.351        *          1.88      k= 7, v=18
--------------------------------------------------------------------------
s =  131.177
Note: df used for table values are approximate when v > 20.

Estimates of EC%
--------------------------------------------------------------------------
Parameter   Estimate      95% Bounds           Std.Err.   Lower Bound
                        Lower     Upper                    /Estimate
EC5            3.9         3.3       4.6         0.034        0.85
EC10           4.3         3.7       4.9         0.030        0.87
EC25           4.9         4.4       5.5         0.024        0.89
EC50           5.7         5.2       6.3         0.020        0.91

        Slope =    9.88  Std.Err. =    1.39


Goodness of fit: p =    0.090  based on DF=        5.0       18.
--------------------------------------------------------------------------
5819GC : Area under the growth curve, mg/L; 0-70.5 hours
--------------------------------------------------------------------------
Observed vs. Predicted Treatment Group Means
--------------------------------------------------------------------------
    Dose     #Reps.     Obs.      Pred.      Obs.      Pred.     %Change
                        Mean      Mean      -Pred.    %Control

    0.00      5.00      905.   1.04e+03     -135.      100.       0.00
    0.100     3.00      981.   1.04e+03     -58.6      100.     2.19e-14
    0.200     3.00   1.21e+03  1.04e+03      174.      100.     2.19e-14
    0.500     3.00   1.14e+03  1.04e+03      99.5      100.     2.19e-14
    1.00      3.00   1.03e+03  1.04e+03     -8.73      100.     3.46e-12
    2.00      3.00   1.06e+03  1.04e+03      19.0      100.     0.000315
    5.00      3.00      750.      750.    -0.00141     72.1      27.9
   10.0       3.00      8.83      8.83    0.000137    0.850      99.2
```

CETIS Summary Report

OCSPP 850.4500 Algal Toxicity   **Water Quality Institute**

Batch ID:	07-3045-7687	**Test Type:**	Algal Cell Growth (96-h)	**Analyst:**	M. Lowitt
Start Date:	13 Aug-91	**Protocol:**	OCSPP 850.4500 Aquatic Plant (Algae)	**Diluent:**	
Ending Date:	16 Aug-91	**Species:**	Nitzschia palea	**Brine:**	
Duration:	72h	**Source:**	Not known	**Age:**	NR

Sample ID:	13-3416-4544	**Code:**	47135819	**Client:**	Cambridge Environmental, Inc.
Sample Date:	13 Aug-91	**Material:**	Denatonium benzoate	**Project:**	Mammal Repellant
Receive Date:		**Source:**	Johnson Matthey Macfarlan Smith		
Sample Age:	NA	**Station:**			

Batch Note: 70.5 hr test
Sample Note: 70.5 hr test

Comparison Summary

Analysis ID	Endpoint	NOEL	LOEL	TOEL	PMSD	TU	Method
10-9748-4809	72h Absorbance	5	10	7.071	43.6%		Dunnett Multiple Comparison Test
00-6896-7546	72h Absorbance	5	10	7.071	31.2%		Williams Multiple Comparison Test

72h Absorbance Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Min	Max	Std Err	Std Dev	CV%	%Effect
0	Negative Control	5	26.3	23.8	28.8	23.2	28.2	0.898	2.01	7.63%	0.0%
0.1		3	37.7	18.6	56.8	29	43.5	4.44	7.69	20.4%	-43.5%
0.2		3	32	14.5	49.6	26.2	39.9	4.08	7.07	22.1%	-21.8%
0.5		3	32.3	20.3	44.2	29.2	37.8	2.77	4.8	14.9%	-22.7%
1		3	26.7	19.8	33.6	23.7	29.2	1.61	2.78	10.4%	-1.52%
2		3	35.1	1.75	68.4	27.3	50.6	7.75	13.4	38.2%	-33.5%
5		3	23.7	19.7	27.7	22.5	25.5	0.928	1.61	6.79%	10.0%
10		3	2.69	0.364	5.01	1.64	3.44	0.54	0.935	34.8%	89.8%

72h Absorbance Detail

Group	Control Type	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5
0	Negative Control	26.7	27.8	28.2	23.2	25.6
0.1		29	40.7	43.5		
0.2		26.2	30	39.9		
0.5		37.8	29.2	29.8		
1		27.2	23.7	29.2		
2		50.6	27.4	27.3		
5		23	25.5	22.5		
10		2.98	1.64	3.44		